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14. ABSTRACT

The research focused on complement activation by nanoparticles which have been already tested in cell culture by AFRL/HEPB. Identification of complement activation pathways was carried out by evaluation of the concentration of pathway specific complement split products generated during human plasma exposure to nanoparticles. Measurements were performed using assay kits supplied by QUIDEL Corporation San Diego California. Different sizes of nanoparticles such as silver (Ag; 15, 100 nm), molybdenum (MoO3; 30, 150 nm), aluminum (Al; 30, 103 nm), iron oxide (Fe3O4; 30, 47 nm), and titanium dioxide (TiO2-40nm) were evaluated for their complement activation potential. The complement activation properties of relatively larger particles of cadmium oxide (CdO; 1 µm), manganese oxide (MnO2; 1-2 µm), and tungsten (W; 27 µm) were assessed. Additionally the effects of nanoparticles coated with lipopolysaccharide on complement activator properties were evaluated.

15. SUBJECT TERMS

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EFFECT OF NANOPARTICLES ON COMPLEMENT SYSTEM IN CELL CULTURE MODEL

EORAD GRANT NO GRANT 053008

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Warsaw 15/09/2006

Report:

Introduction:

Unique characteristics of nanoparticles results in a rapidly growing interest of scientific community and industry in the development of a new devices and applications based on nanotechnology. Nanoparticles are also generated during a wide variety of physical, chemical and biological processes. As their physiochemical characteristics are so unique, there is relatively little knowledge about their effect on human organism. The project focused on the evaluation of nanoparticles interaction with biological targets in *in vitro* models which are related to intravenous exposure. This knowledge is a key factor for the future development of nanotechnology based medical applications (cancer therapy, targeted drug delivery etc) .

The contract covered evaluation of the complement activation by a set of nanoparticles and identification of the activation pathways involved. The study was performed on selected nanoparticles varying in composition and size. In addition to complement activation measurements, cytototoxic effects of nanoparticles were measured using different cell systems. To model intravenous administration of nanoparticles, the effect of compounds on cultured human primary endothelial cells were evaluated. To determine the specificity of observed reactions, obtained results were compared with the effect of nanoparticles on 3T3 cells and primary human fibroblasts.

Short description of the project

The first phase of the project included collection of materials and preparation of testing methods. Protocols were written for the complement activation and cytotoxicity tests. For the purpose of the project the primary cultures of human umbilical cord endothelial cells were established. Endothelial cells together with complement based tests formed a battery of tests for the assessment of blood and vessels compatibility of nanoparticles.

For the complement activation test human serum from volunteer donors was collected, characterized for the basic activation and stored for future use. ELISA kits for the evaluation of the complement activation were ordered, delivered and checked for performance.

In the second phase of the project, complement activation properties of the selected set of nanoparticles were evaluated. Nanoparticles selected for the study differed with chemical composition, size and organization. In parallel cytototoxic effect of nanoparticles on human primary endothelial cells were evaluated. Technical problems associated with the novelty of the tested materials required to redesign our standard procedures to avoid erroneous results caused by incompatibility with testing material. This resulted in creation of the a modified procedure for cytotoxicity testing of nanoparticles in *in vitro* systems. Additionally cytototoxicity tests were performed on fibroblasts (both human primary fibroblasts and cell line 3T3).

Results of the study

As a result of the study the 11 nanoparticles were characterized for their biocompatibility in blood vessels environment using *in vitro* system.

Obtained results indicate that:

1. Nanoparticles toxicity in vitro can't be measured using methods which were designed and/or validated for chemicals
    a. Optical based measurements should be modified to avoid interference of nanoparticles with optical measurements
    b. Effect of nanoparticles interaction (chemical and/or physical) with indicatory dye have to be taken into consideration.
2. Results of the cytototoxicity tests based on fibroblasts tend to underestimate potential toxicity of nanoparticles in case the intravenous administration is planned.

3. Toxic effect of tested nanoparticles is size dependent and decreases with the increase of size.
4. Complement activation by nanoparticles is dependent of their surface composition.
5. Complement activation water suspensions of tested nanoparticles are causing any significant, except silver nanoparticles.
6. 35 nm silver nanoparticles a more potent activators of complement than 25 nm and 10 nm silver nanoparticles.
7. Activation of complement on silver nanoparticles involves only alternative pathway.
8. Complement activation by silver nanoparticles might be decreased by the 12 h preincubation with water (the effect is probably caused by chemical reaction of silver water).
9. Capacity of nanoparticles to bind LPS varies between tested nanoparticles
10. Diamond nanoparticles are not accumulating LPS on their surface
11. Fullerenes, Hematite, and aluminum oxide nanoparticles are strong activators of complement when coated with LPS.

Practical considerations

1. As a result of the project, modification of the cytotoxicity test system, compatible with this nanoparticles, have been established. The same strategy can be used to adopt other cyctotoxicity tests system for nanoparticles testing.
2. Endothelial cells are valuable target in case of toxicological studies of nanoparticles, specially when intravenous applications are considered. Fibroblasts can't be used as a target cellular system as this might result in underestimation of toxicity.
3. Complement mediated toxicity is caused only marginally by the intrinsic property of the tested nanoparticles (i.e. materials included in thestudy), but by their capacity to bind and expose exogenous substances (in the case of our study: bacterial LPS)
4. Differences in LPS binding of different nanoparticles should be taken into consideration when exposure to nanoparticles is foreseen.
5. Carbon (and diamond) coating seems a promising strategy to increase biocompatibility of nanoparticles.

Planned dissemination of the results

Selected results of the project will be presented during the Conference of the European Association of Toxicology in Vitro (Ostend 2-5 October 2006) and subsequently published in peer review journal. It is planned that methodological improvements developed during the study (MTT assay on nanoparticles) will be also published (Journal of Immunological Methods).

Problems encountered during the project

The work started with almost four weeks after stated in the contract because of the financial procedures required to cash US checks in Poland. Additional six weeks delay was caused by the problems associated with the shipment of nanoparticles from US (Nanotechnologies, Inc. Texas). Aluminum nanoparticles were not shipped to Poland because of the very complicated safety procedures (labeled as flammable and explosive).

Materials and methods

Tested nanoparticles

Eleven different nanoparticles preparations were evaluated in the project.
All of them formed suspension in water. Selected nanoparticles differed in composition and size. Selected set contained silver nanoparticles of three different sizes (10 nm,25nm and 35 nm). Five different nanoparticles with carbon surface (Fulerene C60, Iron coated with Carbon, Nanotubes (two sizes) and Diamond nanoparticles) and three nanoparticles composed of oxidized metals (Magnetite and aluminum oxide (2 sizes) were also tested.

	Name	Internal code	Size	Supplier and cat no.:
1	Fullerene C60	Fu60	0.6 nm	Sigma: 572500
2	Iron coated with Carbon	FeC	25 nm	Nanotechnologies Inc: Fe-25-ST2 M19004
3	Magnetite	Fe_30_4	25 nm	Nanotechnologies Inc: Fe3O4-25 M14010
4	Aluminium Oxide	Al_2O_3 30	30 nm	Nanotechnologies Inc: Al2O3-30 M1056
5	Aluminium Oxide	Al_2O_3 40	40 nm	Nanotechnologies Inc: Al2O3-40 M1049
6	Silver	Ag 10	10 nm	Nanotechnologies Inc: Ag-10-ST2-B M4049B
7	Silver	Ag 25	25 nm	Nanotechnologies Inc: Ag-25-ST2 M4072
8	Silver	Ag 35	35 nm	Nanotechnologies Inc: Ag-35-ST3 M4068
9	Diamond	Dia	3.5-6.5 nm	Sigma: 636428
10	Multiwall carbon nanotubes	NTB	OD=10-20nm ID=5-10nm, length=0,5-200um,	Sigma: 636525
11	Multiwall carbon nanotubes	NTB	OD=40-60nm, ID=5-10nm, length=0,5-500um	Sigma: 636622

The selection was based on the potential use in medicine and ability to form a suspension when mixed with water (required when intravenous application is considered).

Name	Potential application in medicine
Fullerene C60	Contrast agent, drug development [1, 2]
Iron coated with Carbon	Imaging [3] Cancer therapy [4]
Magnetite	Magnetic field targeted dug delivery [5]
Aluminium Oxide	Drug carriers, nanosensor [6]
Silver	Nanoprobes [7]
Diamond	Biomaterials coating
Multiwall carbon nanotubes	Microsensors [8]

Literature:

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Cells:

- Primary endothelial cells isolated from human umbilical veins
- Primary human skin fibroblasts
- Balb/C 3T3 Clone 31 ECAC cell line

Plasma:

- Human isolated plasma

Testing kits:

- IC3b ELISA test kit QUIDEL San Diego USA
- Bb ELISA test kit QUIDEL San Diego USA
- SC5-9 ELISA test kit QUIDEL San Diego USA

Primary endothelial cells isolated from human umbilical veins

Aseptically taken umbilical cord, preferably abort 20-30cm length was clamped and dipped in 70% ethanol for 30s. Umbilical cord (vein) was washed with PBS to remove blood.
Collagenase/dispase solution (Collagenase (type I – 1mg/ml (242U/ml)) and dispase (2mg/ml (about 1,4U/ml)) solution in Dulbecco's modified Eagle's medium (DMEM – Gibco) containing antibiotic/antimicotic). was run into vessel until it appeared at bottom end. Bottom end was clamped and more solution was added to the lumen of vessel. Umbilical cord was incubated at 37ºC for 30 minutes (for time to time the cord was rotated). The cell suspension was collected in 50ml centrifuge tube. Lumen of vessel was rinsed with PBS and added to cell suspension. Pooled digest was centrifuged at 200g for 10 minutes.Cells were washed twice and centrifuged. Final pellet was resuspended in culture medium (DMEM:F-12 (1:1) with Glutamax I, antibiotics/antimicotics, 20% FBS, 18U/ml heparin, 10ng/ml EGF) and seeded into flasks. Cells were seeded on collagen coated plastic at conc. 10^4 per well in 96 well plates. After 24 h of adaptation, cells were exposed to the range of concentrations of nanoparticles.

Human primary fibroblasts cultures

Anonymysed human material from the surgical wastes was used for the isolation of skin fibroblasts. Surgical waste from the plastic surgery of the breast were used as a source of cells used in our experiments. The epidermal layer was detached mechanically from dermis after 18 hours incubation in 0,5% dispase at 4ºC (Gibco cat no. 17105-041). Fibroblasts were obtained from the skin dermis remained after removal of the epidermis. Dermis was cut into 2 x 2 mm pieces and transferred to a 25 cm2 flask. Pieces were incubated at 37ºC for 48 hours in 5 ml of Dulbecco-modified Eagle's Medium DMEM (Gibco cat no. 31885-023) with 10% FBS (Gibco cat no. 10106-169) and crude collagenase (Gibco cat no. 17101-015). The final concentration of collagenase was 200 Units/ml. After complete disaggregation of the tissue the supernatant was centrifuged and cells were collected. Fibroblasts were resuspended in 5 ml of DMEM with 10% FBS, 1% L-glutamine (Gibco cat no. 25033-010) and 1% Antibiotic-Antimycotic and seeded in 25 cm^2 flasks. After 4 passages cells were seeded on 96 well plates and exposed for 24 h to the array of concentrations of tested compounds. After exposure cell response is measured using MTT test.

Preparation of human plasma

Blood was obtained from healthy volunteers. Plasma was separated by centrifugation centrifuge (400g, 10min) of heparinised (10 U/ml) (S-Monoovette Sarstedt,Germany) freshly-obtained blood. The separated plasma was then centrifuged once again (800 g 10 min), aliquoted in 2 ml polypropylene tubes and stored in -70ºC. The processing time was never longer than 1 h.

Preparation of nanoparticles suspensions

Nanoparticles were weighted in labelled polypropylene tubes. For complement activation tests nanoparticles were suspended in EBSS (with Ca and Mg). For cell culture experiments nanoparticles were suspended in cell culture medium. Prepared suspensions were agitated with ultrasounds for 20 minutes at 37C, then stored at +4C and used for testing on the following day. Stock suspensions were prepared at concentrations 10 mg/ml. On the day of experiment, stock were sonicated for 10 minutes to obtain uniform suspensions and subsequently diluted ten times to obtain testing suspension at concentration 1 mg/ml.

Complement activation assay

Human serum was exposed to the tested substances in 2ml polypropylene vials for 60 minutes. Exposure is conducted in a standard CO2 incubator (5% CO2, 37°C). Each sample was tested in triplicates. After exposure samples were cooled on ice and mixed with the cocktail of enzyme inhibitors (Sample Stabilizing Solution) to prevent future activation. Samples were then stored at -70°C for future analysis. Generation of the complement activation products were measured using three different ELISA systems (iC4b, Bb, SC5-9; QUIDEL® San,Diego,CA,USA).

LPS exposure experiments

LPS exposure
LPS stock (1mg/ml of water) was resuspended using bath sonicator, for 10 min.
10 µl of LPS stock was added into 90 µl of nanoparticles suspension (1 mg/ml)
Control sample (without LPS) was prepared by adding 10 µl of EBSS into 90 µl of nanoparticles suspension (1 mg/ml). Samples were incubate overnight on a shaker at room temperature.

On the following day samples were washed twice with EBSS (by centrifugation) to remove unbound LPS and resuspended in 100 µl of EBSS.
Thawed human plasma was aliquoted of into 2 ml polypropylene vials (90µl/vial). Plasma was then exposed to the tested samples by addition of 10µl of prepared suspensions containing 0,9 mg/ml of nanoparticles and 1 µl of LPS .
Samples were incubated for 60 minutes in CO2 incubator.
Measurements of the complement split products in samples were performed on samples diluted 1:40 in Sample Diluent (add 8µl into 280µl of Sample Diluent) using ELISA system.

MTT cytototoxicity assay

After two passages, cells were seeded on 96 well plates at concentration $2x10^4$/well and exposed to the array of concentrations of tested compounds. After exposure cell response was measured using MTT test.
The MTT test was performed on two plates in parallel. One plate contained exposed cells and the second contained only culture medium (50µl /well) with tested concentrations of nanoparticles (50µl /well). After exposure 50µl /well of MTT stock solution (1,5 mg/ml) were added to both plates. The final concentration of MTT was 0,5 mg/m in each well. After 3 hours incubation both plates were centrifuged and supernatants were removed. Formed formazan crystals were solubilization using DMSO (150µl/ well). The plates were incubated on the shaker for 20 minutes. After solubilization of formazan crystals, plates were centrifuged again and from each well 100 µl of supernatant were transferred to prepared empty 96 well plates. Both plates were read in a plate reader using two filters (570nm and 650 nm).
Obtained results of the 650nm readings were subtracted from the results of 570 nm readings for both plates. Finally results of the optical density of wells congaing only nanoparticles were subtracted from the optical densities of the cell exposed plate.
The toxic effect of nanoparticles was calculated as percentage of the optical density in exposed well compared to the control wells (100% viability)

Remarks:

<u>Two crucial elements introduced to the of the MTT cytototoxicity test were:</u>

1) *The use of two plates exposed to the tested concentrations of nanoparticles. One plae contained target cells, while the second only nanoparticles. The second plate was used for the detection of formazan generation by interaction of MTT with nanoparticles.*
2) *The use of supernatant from the plates which were not containing nanoparticles. This enabled to avoid errors associated with nanoparticles interference with optical density measurements.*

Results

Complement activation model

Normal human plasma was exposed to nanoparticles for the assessment of complement activation potential of nanoparticles. Methodology was based don ISO procedure used for biocompatibility assessment of biomaterials in contact with blood. Tested nanoparticles were suspended in water and then exposed to human plasma. As a indicator of complement activation iC3b fragment release was measured. 11 different nanoparticles were tested. Obtained results indicate relatively low capacity of tested nanoparticles to activate complement system. This capacity was significantly increased when exposure was performed immediately after nanoparticles suspension was formed. This effect was specially important in case of silver nanoparticles (probably because of chemical interaction between silver and water). The most interesting was that in case of silver the complement activation was not linearly related with the size of the tested nanoparticles, being the highest in case of the biggest tested nanoparticles (35 nm).

Results obtained using Bb fragment generation as an end point indicated that observed activation was mediated by the alternative pathway (no complement activation by classical pathway was observed).

It can be concluded that complement activation should be taken into consideration in case of nanoparticles which might be in contact with human organism.
The capacity of the tested nanoparticles to activate complement was relatively low and the risk of adverse reactions might be considered as marginal.

The situation is totally different in case of nanoparticles coating with lipopolisaccharide. In this case complement activation considerably differs between nanoparticles, being the highest in case of fullerene, ferric oxide and aluminium oxide. Which is very interesting no activation was observed on LPS treated diamond nanoparticles, which might indicate their potential usefulness for coating of medical devices which are in contact with blood.

Modification of the MTT test

Standard protocol for the MTT test was modified to enable proper evaluation of toxic effect of nanoparticles in cell culture conditions. Preliminary experiments revealed that the optical and chemical properties of nanoparticles might cause errors in the assessment of toxicity using cytototoxicity tests base on optical density measurements. Some of the nanoparticles are able to interfere with the substrate (MTT) increasing amount of formazan formed, even in the absence of living cells (fig1.).

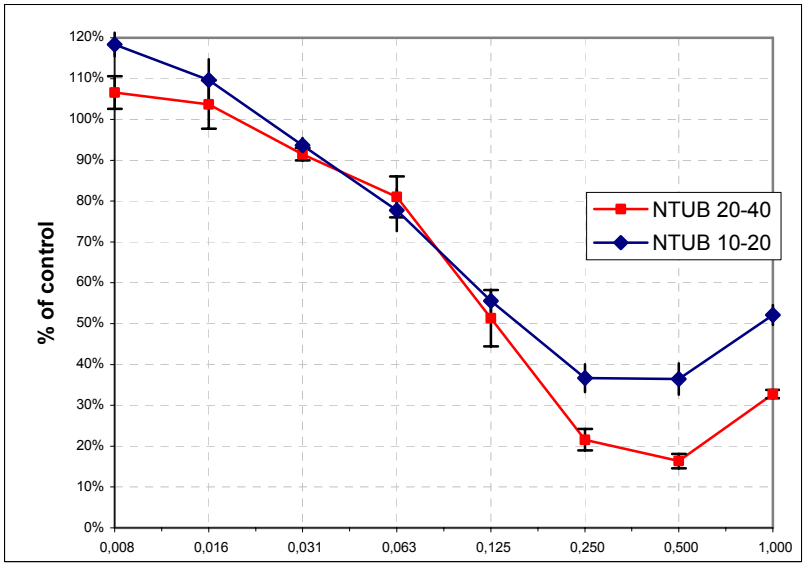


Fig. 1 The results of the MTT test of Mutiwalled nanotubes (NTUB) on Human umbilical cells in the unmodified test. Visible false “increase” of viability at high concentrations of nanoparticles. This phenomenon is frequently reported in the literature and wrongly interpreted as a cause of nanoparticles aggregation which causes decreased availability to target cells.

To overcome this limitation, the test has been modified by addition of a second plate where nanoparticles were incubated with MTT in the absence of cells. Optical densities from this plate were subtracted from the optical densities obtained from the cell seeded plate.

The other limitation of the optical density based test is direct influence of nanoparticles on the optical characteristics of the well.

To eliminate this limitation the plates were centrifuged and supernatant (free of nanoparticles) was transferred into the second plate (on which the OD measurements were performed). Obtained optical densities were subtracted.

The results of the modified procedure are shown on fig.2. It is clearly visible that the modification enabled to eliminate the error caused by nanoparticles interference with the system

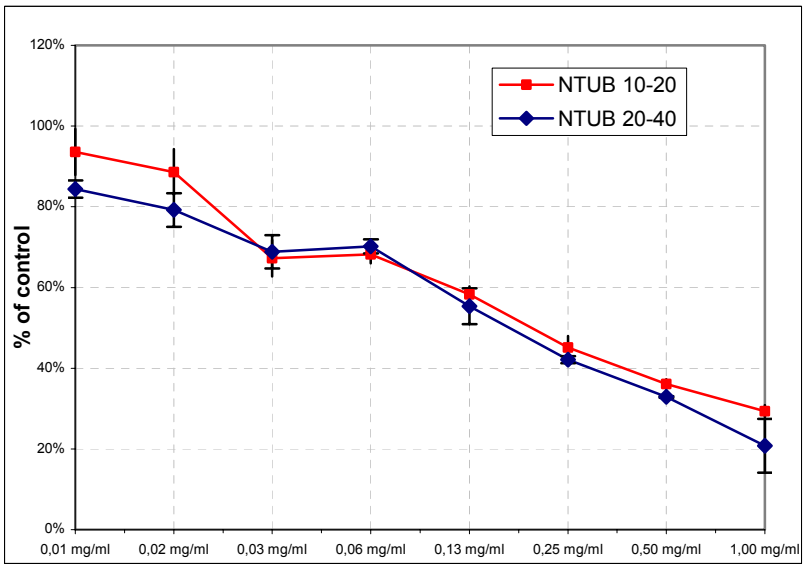


Fig. 2 The results of the MTT cyctotoxicity test on human umbilical endothelial cells in the modified test performed on two plates. False "increase" of viability at high concentrations of nanoparticles was eliminated.

Results of cyctotoxicity studies on different target cells

The modified MTT test was used for the assessment of nanoparticles toxicity in three different cell cultures: Human primary endothelial cells and human primary fibroblasts. Additionally tests were performed on Balb/C mouse 3T3 cell line. Maximum tested concentration of nanoparticles was 1 mg/ml.
Results obtained in the study are represented in the table:

	Name	Human endothelial cells IC50	Human fibroblasts IC50	3T3 cell line IC50	Size
1	Fullerene C60	>1mg/ml	>1 mg/ml	>1mg/ml	0.6 nm
2	Iron coated with	0.25 mg/ml	0.45mg/ml	0.52 mg/ml	25 nm

	Carbon				
3	Magnetite	0.2 mg/ml	>1 mg/ml	>1mg/ml	25 nm
4	Aluminium Oxide	0.7 mg/ml	>1 mg/ml	>1 mg/ml	30 nm
5	Aluminium Oxide	0.7 mg/ml	> 1mg/ml	>1 mg/ml	40 nm
6	Silver	0.015 mg/ml	0.08 mg/ml	0.06 mg/ml	10 nm
7	Silver	0.05 mg/ml	0.17 mg/ml	0.18 mg/ml	25 nm
8	Silver	0.04 mg/ml	0.27 mg/ml	0.33 mg/ml	35 nm
9	Diamond	0.16 mg/ml	>1mg/ml	>1 mg/ml	
10	Multiwall carbon nanotubes	0.18 mg/ml	>1 mg/ml	>1 mg/ml	OD=10-20nm ID=5-10nm, length=0,5-200um,
11	Multiwall carbon nanotubes	0.18 mg/ml	1mg/ml	>1 mg/ml	OD=40-60nm, ID=5-10nm, length=0,5-500um

Both human and mouse fibroblasts gave the same classification of toxicity of the tested nanoparticles (Fig. 3). High correlation (Spearman r=1 p <0.05) between results obtained using those two tests indicate that there 3T3 cell line is a good target for the assessment of the basic cytototoxicity and there is no need for human primary fibroblasts.

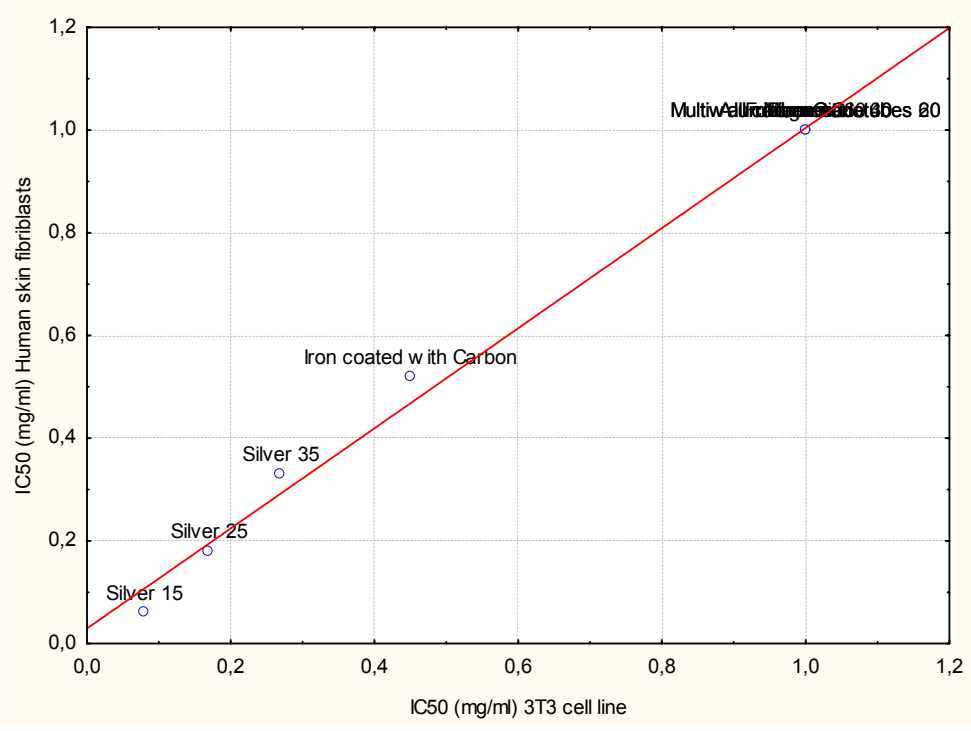


Fig. 3 Correlation between IC50 values obtained using 3T3 cell line and promary fibroblasts

Obtained results indicate that Human primary endothelial cells are the most sensitive target cells. The biggest difference in fibroblast and endothelial cells toxicity was observed in case of Multiwall carbon nanotubes and diamond.
All tested systems identified fullerenes C60 as non toxic agent.

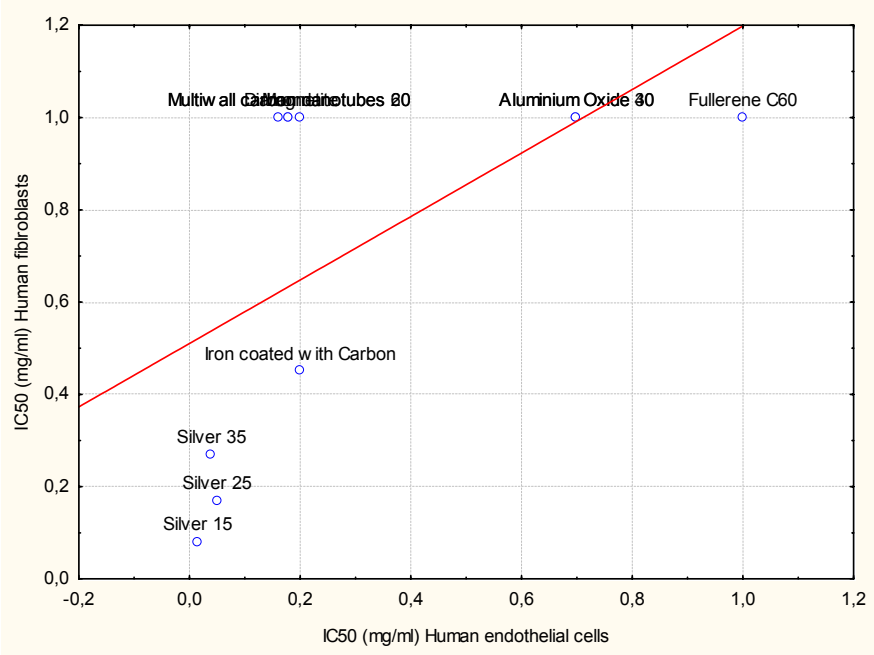
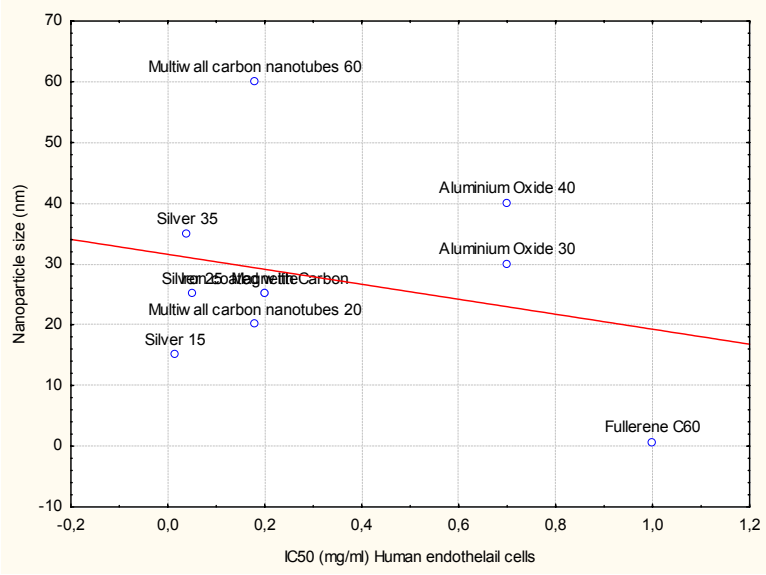
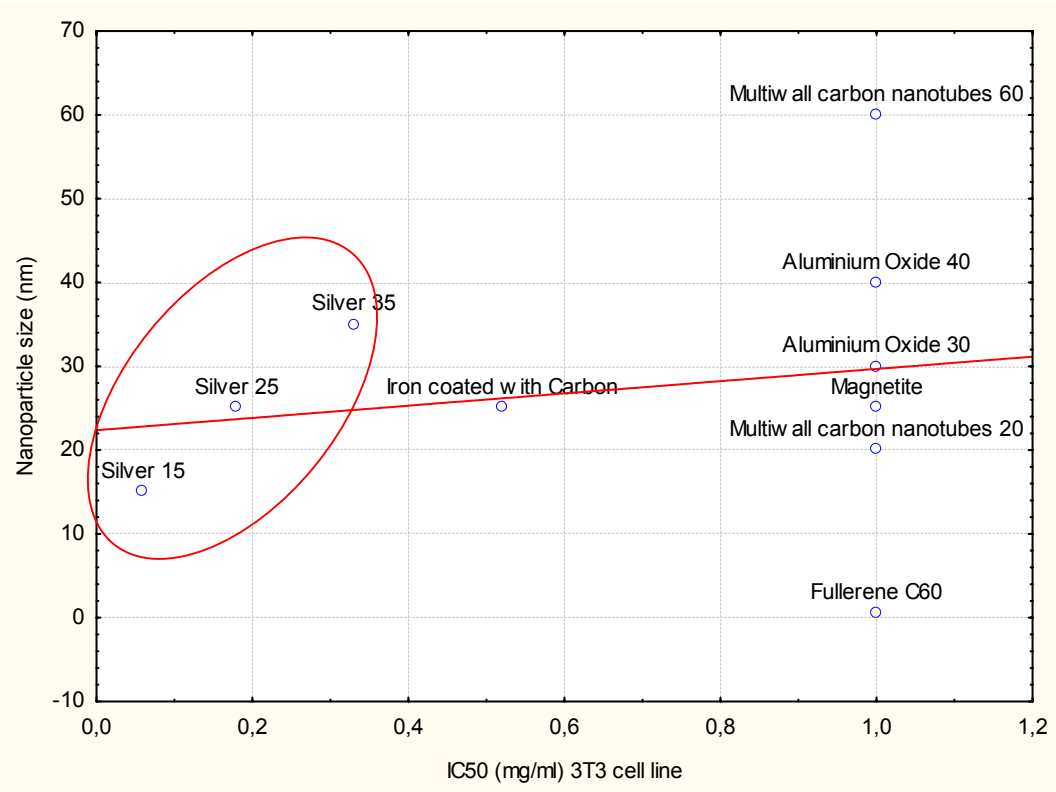


Fig. 4. Results form the experiments performed on the human endothelial cells are doffernet from those obtaine on fibroblasts. Endothelial cells are the primary target for most of the tested nanoparticles. Nanoparticles with IC50>1 were indicated as 1 mg /ml. (speraman r=0.71 p<0,05)

The toxic effect on cells is more related to the chemical properties of the material than to the size of nanoparticles.



Corelation between size and IC50 can be found only in case of similar nanoparticles (ie silver).



Complement activation by nanoparticles

of the complement activation products are then measured using four different ELISA systems. Performed experiments revealed that for highly reactive nanoparticles activation of complement is dependent on time of incubation of nanoparticles with water. The example are 10 nm silver nanoparticles which have very high capacity of complement activation (similar to LPS) when added directly to human plasma. All protocols were modified to overcome this effect, by 12 h preincubation of nanoparticles in water suspension at +4C. After this time nanoparticles for all experiments were washed once in EBSS and resuspended. Activation of complement by the tested nanoparticles was relatively low and most nanoparticles were not activating complement more than the sponateous (control) activation (Studend T test $p<0,05$). Only silver nanoparticles were significantly more potent as complement activators (See Fig below)

Activation was identified as alternative pathway, as Bb fragment generation pattern was correlated with the pattern of iC3b.

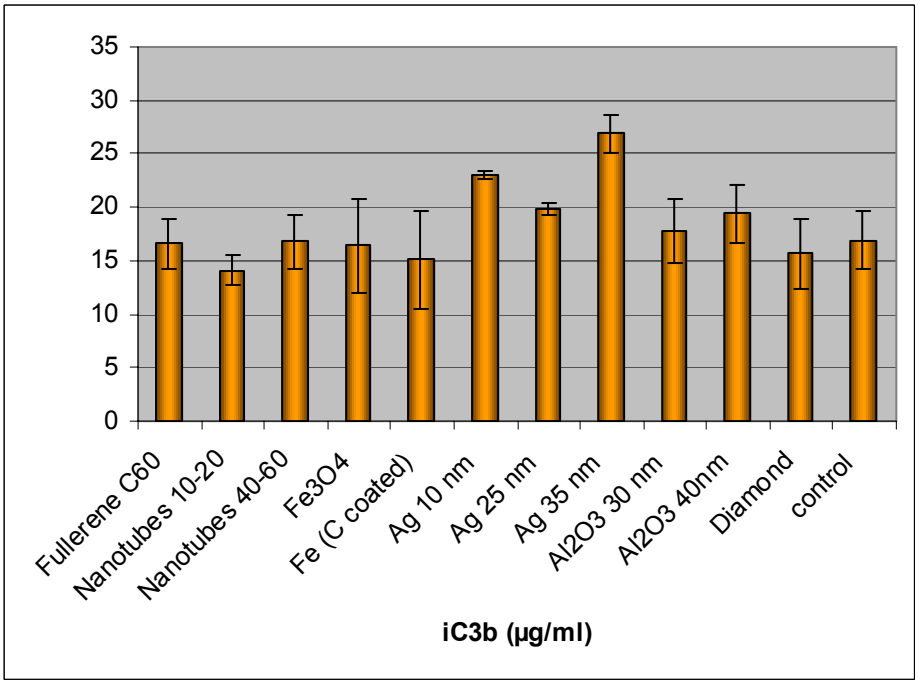
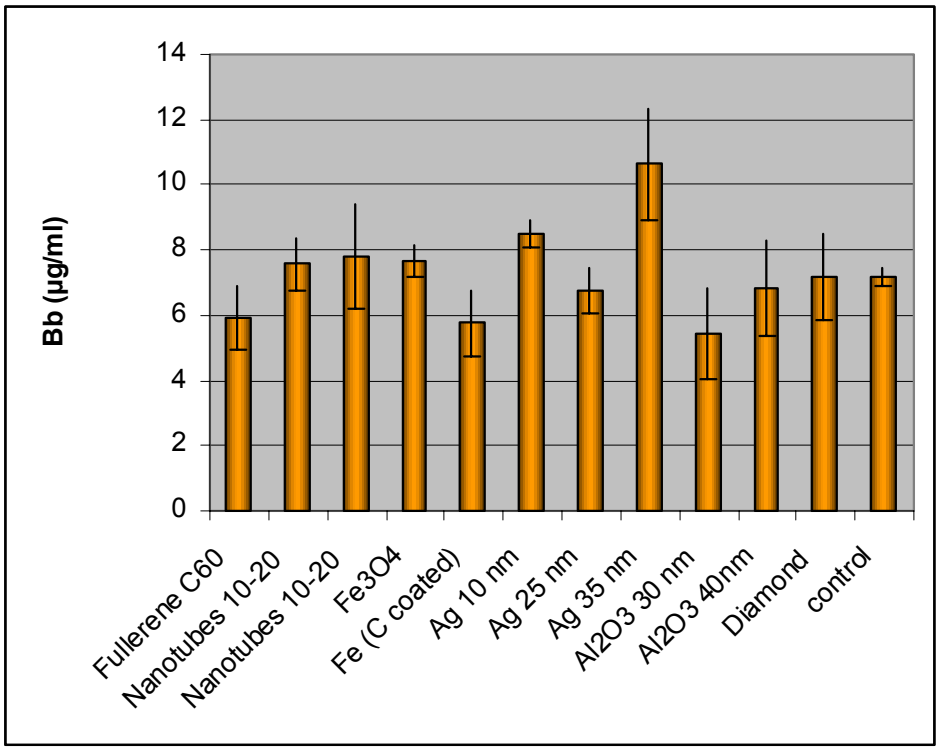
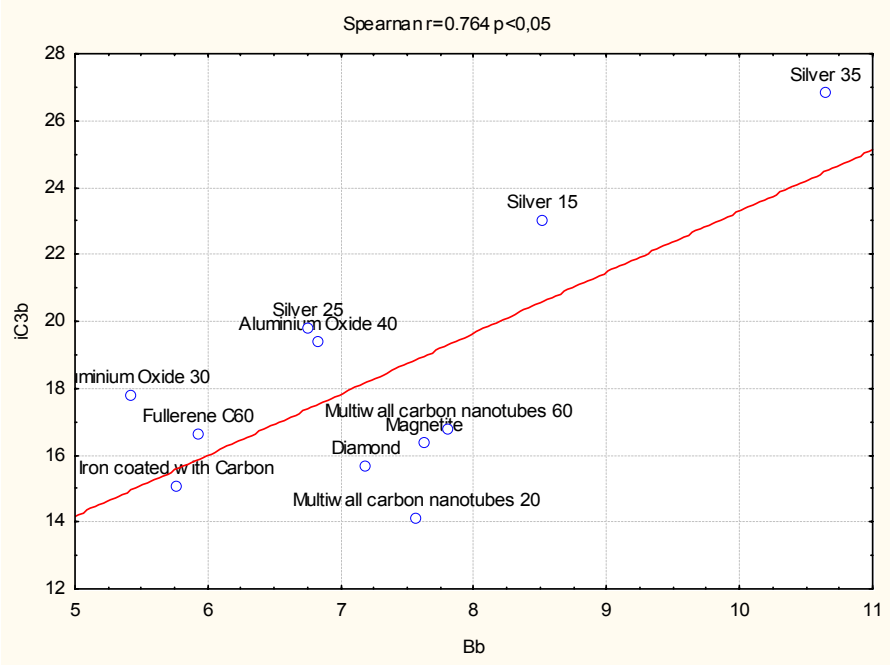


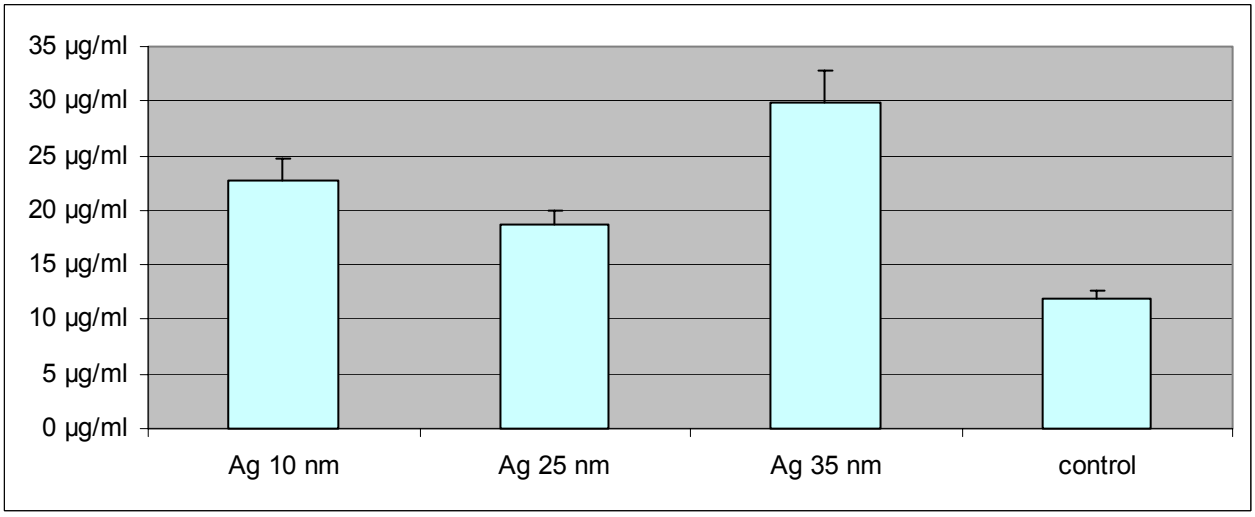
Fig. 1. Activation of complement on LPS exposed nanoparticles.





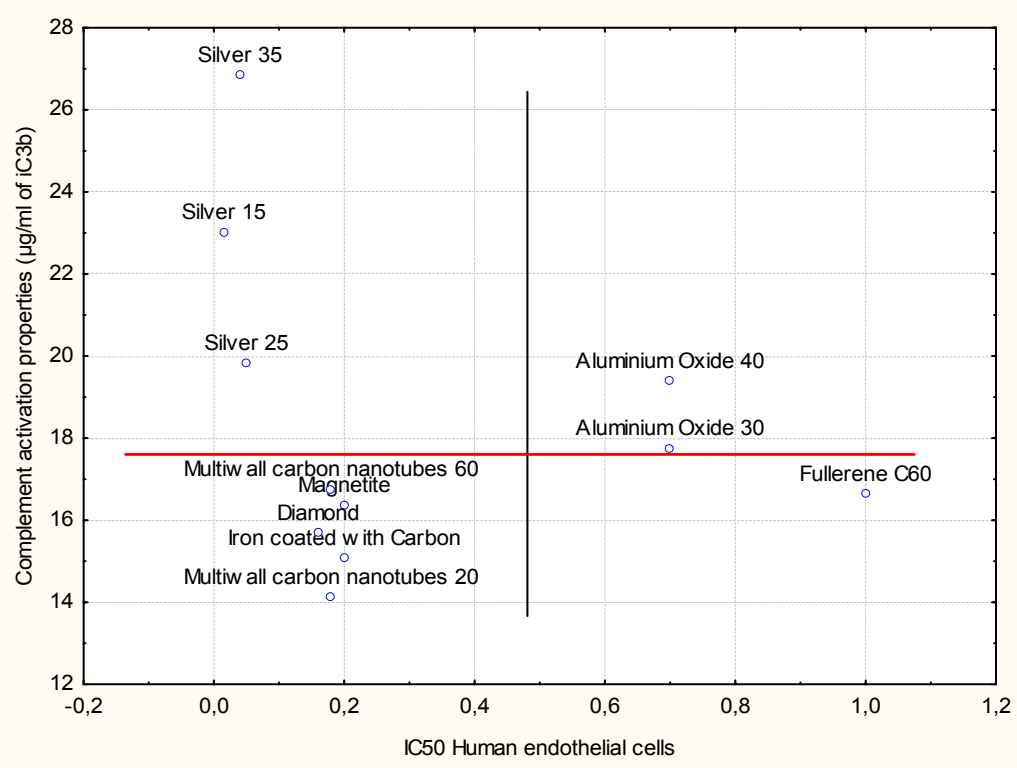
It can be concluded that from the tested nanoparticles only in case of silver nanoparticles, complement mediated toxicity should be taken into consideration.

The most potent activators of complement were 35 nm silver nanoparticles, thaen 10 nm and lastly 25 nm sized silver nanoparticles.

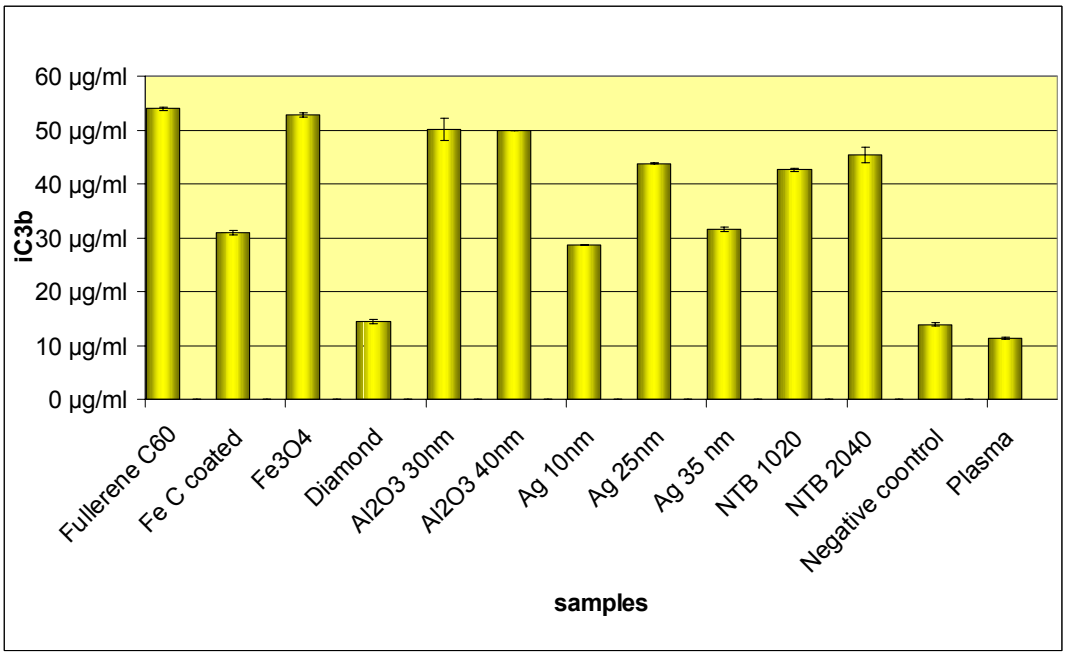


Comparison of cell toxicity measured usinf human endothelial cells and complement activation capacity indicates that from the set of tested nanoparticles only fullerens are no toxic to any target cells and have no capacity to activated human complement. It can be concluded that fullerens C60 should have a very low toxicity when injected intravenously, which

seems very important as this material has great potential as element of drug delivery systems.



LPS adsorption on nanoparticles revealed that diamond and carbon coated ferrous nanoparticles have very low potency to activate complement after contact with LPS. In case of diamond nanoparticles no activation was observed.



This phenomenon might be used in practice. Nanoparticles coated surfaces of medical devices should not accumulate LPS (less complement activation and lower Pyrogenicity). The results are very important as airborne nanoparticles are frequently opsonised with fragments of bacterial walls (LPS, LTA). Even on case of low cytotoxicity of nanoparticles, it's complement activation capacity might trigger inflammation when contaminated with LPS (the same principle applies to fullerene C60).

Final remark:

The project resulted not only in better characterisation of the toxicological profiles of several nanoparticles which might be potentially used in medicine and basic principles of nanoparticles toxicity mechanisms. Better understanding of differences between chemical testing and nanoparticles testing *in vitro* resulted in development of a new specialized testing systems (i.e. MTT test for nanoparticles testing) which can be in future used in validation studies.

With best regards

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